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GRANT NUMBER DAMD17-94-J-4032

TITLE: Breast Cancer Cell Metabolism Studied by MRS

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REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jul 96 - 30 Jun 97)

4. TITLE AND SUBTITLE

Breast Cancer Cell Metabolism Studied by MRS

5. FUNDING NUMBERS

DAMD17-94-J-4032

6. AUTHOR(S)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

It is proposed to study the metabolism of intact breast cancer cells using non-invasive magnetic resonance spectroscopy (MRS) methods. Magnetic resonance imaging (MRI) has been shown to have some value in assessing breast lumps, but lacks specificity. MRS can in principle provide this specificity. In order to evaluate the potential of MRS, systematic studies will be carried out on cell lines selected for the progressive estrogen independent phenotype and the effects of hormones and tamoxifen.

The cell studies will provide basic information on the metabolic profile of different BC cell lines in the progression from estrogen and drug sensitive to estrogen independent and drug resistant phenotypes. Cells are embedded in gels and are examined in the MR spectrometer while undergoing perfusion. Previous studies have mainly used carbohydrate (agarose) gels. But, based on preliminary results, we will use a protein gel (Matrigel) for which the cells have surface receptors, so that their metabolism can be monitored while they are proliferating. This allows the effects not only of different phenotypes, but also of hormones, and anti-estrogens such as tamoxifen, as well as certain drugs and drug combinations to be evaluated. Both ^{31}P and proton MR methods will be applied. The results will be of significance not only to understanding the fundamental biological processes involved, but also to the observation of in vivo MRS of breast lumps.

14. SUBJECT TERMS
Breast Cancer

NUMBER OF PAGES 18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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7/28/97

7/14/97

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1. INTRODUCTION

A. Scientific Background

We are investigating the metabolism of intact breast cancer cells using magnetic resonance spectroscopy (MRS) methods in order to elucidate the processes involved in the development and possible treatment of breast cancer.

Magnetic resonance techniques have become important in clinical imaging, but perhaps less well known is the fact that MRS can be used as a research tool to study the metabolism of isolated intact cells (Daly and Cohen, 1989). Such studies can provide important information on biochemical processes, and can be used for the identification of signals and the understanding of metabolic processes *in vivo.*

Perfused intact cells represent possibly the best approach to the non-invasive study of metabolism. In contrast to the *in vivo* situation, the cells are homogeneous, particularly when grown in culture conditions. Such *ex vivo* ^{31}P MRS studies have provided information on normal cellular energetic status, substrate utilization and metabolic pathways, phospholipid pathways, intracellular pH changes and membrane permeability. Using these methods, significant metabolic differences between cell lines have been delineated and effects on metabolism following manipulation with nutrients, hormones, drugs, growth factors, and hyperthermia have been monitored (for a review see Cohen et al, 1995).

A variety of methods for restraining cells for MRS studies of metabolism are currently available. An appropriate technique uses agarose threads; the advantages of this method are: (a) it is a simple, inexpensive and quick technique to use; (b) a large number of cells can be maintained in good metabolic status for prolonged time periods (24 to 36 h); (c) the matrix itself (as opposed to the use of beads) occupies a relatively small volume; (d) effects on metabolism of the addition of metabolite precursors (nutrients) or drugs, or physical changes (such as temperature) can be detected; and (e) both anchorage-dependent and -independent (cancer) cells can be studied. Detailed studies of cell growth and viability (using trypan blue exclusion and cell counting), microscopy, and the measurement of diffusion constants of metabolites (ATP, glucose) and protein content, were previously carried out using cells in agarose threads (Foxall and Cohen, 1983; Foxall et al, 1984; Knop et al, 1984; Lyon et al, 1986; Kaplan et al, 1990) to define this approach.

Agarose is a carbohydrate gel, and in the agarose method cells are "trapped" in the threads and can be removed by gentle pipetting. matrigel is a natural basement membrane and is a protein gel for which breast cancer cells have receptors. It is an extremely open gel that is used extensively for studies of cancer cells (Kleinman et al, 1986), Dispase is used to dissolve matrigel in order to measure cell densities (Daly et al, 1988). It should also be emphasized that a comparison of the same cell line in matrigel cell threads and in tumor xenografts in nude mice gave almost identical spectra (Daly et al, 1988). We have chosen the matrigel thread method as the most effective one for this work, particularly where effects of growth and hormones are concerned. Therefore, the bulk of this proposal is devoted to ^{31}P MRS studies of perfused intact breast cancer cells embedded in matrigel threads.

In order to understand the role of estrogen receptor (ER) status in breast cancer cells Clarke et al. (1989, 1990), have developed special cell lines (see Table 1). The cell lines to be studied in this work are all variants that have been isolated and extensively characterized, and exhibit specific phenotypic changes that reflect critical characteristics of the progression in therapy from hormone sensitive to insensitive phenotype. The ability to detect specific metabolic changes associated with phenotypic changes is substantially

increased, since several of these cell lines were derived from the same parental cell line (MCF-7).

In breast cancer cells and other malignant cell lines, some of the cellular effects of "anti microtubule" drugs are modulated by the action of steroids, estrogens and antiestrogens, and vice versa. For example, colchicine was reported to inhibit translocation of estradiol receptors and the synthesis of progesterone receptors in MCF-7 cells (Parikh et al, 1987); and to sensitize the activation of large conductance chloride channels in fibroblasts upon exposure to extracellular antiestrogens (Hardy and Valverde, 1994). Tamoxifen increases the cytotoxicity of vinblastine in MCF-7 variants which express gp170 (Leonessa et al, 1994) and reversed vinblastine resistance in the mdr1 transfected lung cancer cell line, S1/1.1 (Kirk et al, 1993). The antiestrogen toremifene, 'resensitizes' MDA-MB-231-A1, an estrogen receptor negative breast cancer cells, to vinblastine (Koester et al, 1994). The estrogen metabolite, 17 beta-estradiol glucuronide modulate resistance to taxol and vinblastine in the Dx5 MDR sarcoma cell line (Gosland et al, 1993). It was recently reported that a metabolite of estradiol, 2-methoxyestradiol, inhibits tubulin polymerization by binding to the colchicine binding site on tubulin dimer (Cushman et al, 1995).

Among the anti-cancer drugs that will be examined are taxol, vincristine and adriamycin. taxol (Paclitaxel), a new promising anticancer drug, has recently been approved for the treatment of refractory ovarian cancer, and is showing promising activity in malignant melanoma, breast cancer, and lung cancer (see: Paclitaxel in Cancer Treatment; edited by Mcguire and Rowinsky, 1995). Taxol has demonstrated substantial single agent activity against both minimally pretreated and resistant metastatic breast cancer (Wong and Henderson, 1994). Taxol and vincristine are anti-mitotic drugs that interfere with the assembly process of microtubules. Microtubule functions in cells are highly complex. Aside from serving as passive skeletal supports for the organization of the cytoplasm, their remarkable polymerization dynamics is critical to many of their functions. Drugs like taxol and vincristine interact with microtubule ends and surfaces and modulate the polymerization dynamics. Although all of these drugs bind to tubulin, they exert distinct effects on the protein organization in the cell. In the presence of taxol highly organized bundles of microtubules are formed, while vinblastine induces tubulin self-association to paracrystal formations (for review see: Wilson and Jordan, 1994). These drugs inhibit cell proliferation and replication, induce multi-drug resistance, and have a variety of cellular effects, apparently unrelated to their action on tubulin.

B. Specific Aims

- To characterize the growth of several related human breast cancer cell lines in a natural (protein) basement membrane gel matrigel by ^{31}P MRS spectroscopy, and to compare the role of estrogen receptor (ER) status in ER positive and ER negative cell lines.

- To observe the effects of hormones, such as estrogen and tamoxifen, on the growth and metabolism of these cell lines, and to investigate the effects of drugs, notably taxol, on these cell lines with different ER status and hormone dependence.

- To explore the application of ^{1}H MRS to study cancer cell metabolism with water signal suppression and observation of only intracellular signals, and to investigate the resolved proton signals in breast cancer cell spectra, and in response to the agents described.

2. BODY OF REPORT

A. Cell Perfusion Methods

The use of agarose threads was introduced by Foxall and Cohen (1983; Foxall et al, 1984) and is based on the properties of low-temperature gelling agarose, that allows mixing of cells with liquid agarose at 37°C, and solidification of the mixture at a lower temperature. Cell pellet (1-1.2 ml containing ca 2 x 10^8 cells) are mixed with an equal volume of liquid agarose (1.8%) in phosphate-buffered saline, and immersed in a bath at 37°C for 5-7 min. The mixture is extruded under low pressure through cooled capillary tubing (0.5 mm id) into an MRS tube containing growth medium(for a complete description see Cohen et al, 1989). Using 0.5 mm threads ensures that the threads maintain their mechanical strength and the cells are not metabolically compromised, and are viable and in stable energetic status for more than 24 h. Moreover, it was shown that albumin can readily diffuse into the threads (Kaplan et al, 1990). The gel threads which fill the tube are concentrated at the bottom of the tube by insertion of a plastic insert with the perfusion fittings. The inflow tube is 0.5 mm id, and is placed near the bottom of the tube. The outflow is directed into openings in the insert, and then into an outflow tube. Perfusion rates (0.3-2 ml/min) are maintained by a peristaltic pump, and since the tubes are permeable to air, it is not required to include a gas exchanger in the perfusion system. The perfusion solution should be the buffered growth medium that is most appropriate for the cells studied.

Cells are routinely perfused for periods from 2 to 12 hours, and sometimes much longer if sterility can be maintained (Cohen et al, 1989, Daly et al, 1988). The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that sometimes occurs after ca. 12-24 hours. An initial high level of Pi is indicative of a bad cell sample. If a significant increase of Pi is seen in the first two hours the experiment is usually abandoned. If the Pi/ATP ratio is low, and remains low for this period, the cell sample is considered acceptable (a great deal of experience shows that this parameter is consistent with, but preferable to the exclusion of trypan blue as a measure of cell viability). No other change has ever been seen to occur as long as the cells are adequately perfused. If the perfusion is stopped a rapid increase in the Pi/ATP ratio occurs (Knop et al, 1984). In order to confirm the adequacy of perfusion in any given case the perfusion rate is routinely adjusted to check for no change in the Pi/ATP ratio.

One of the disadvantages of perfusion studies with agarose threads is the limited proliferative activity inside the threads. The gel thread technique was therefore improved by the use of a basement membrane matrix, matrigel, in which anchorage-dependent cells can multiply while being perfused (Daly et al, 1988). Cell pellet (0.1 ml) is mixed with 2 ml of liquid basement membrane, and the mixture is extruded, as described above for agarose threads, into petri dishes. Cells are allowed to grow in the incubator until the desired densities are reached, and the threads are then transferred to a 10 mm MRS tube. Modification of the perfusion apparatus used for the agarose threads procedure, includes the insertion of large capacity filters (20 liter) between the peristaltic pump and the tube, which ensure sterility. Thus, the cells can be perfused with fresh medium for prolonged periods (weeks), at a low perfusion rate of 0.5 ml/min, and MRS spectral changes associated with proliferation can be monitored (Daly et al, 1988). Thus, the use of matrigel allows the expansion of the observation of cell metabolism by NMR methods to a whole range of studies relevant to breast cancer.

B. Proton NMR Cell Studies

Phosphorus-31 NMR methods have proven to be very informative in the study of cellular metabolism (Cohen et al, 1995). However, although proton NMR is intrinsically 14 times more sensitive than ^{31}P NMR, very little research has been done in this area (Cohen et al,

1995). There are three main reasons for this, (a) the overlap of the many signals from hundreds of metabolites, (b) the presence of the huge water signal in biological systems, and (c) the overlap of signals from intracellular and extracellular substances, including buffers, metabolites, etc. A few years ago we solved (b) and (c) by the use of the diffusion-weighting technique, whereby the application of timed pulses allows for the selection of a window for the observation of slowly moving molecules only (Van Zijl et al, 1991). This eliminates all water and extracellular molecules that are moving rapidly, and allowed us to observe the proton signals only from intracellular metabolites.

However, there is still extensive overlap of metabolite signals at 400 MHz, causing difficulties in resolution and assignment of signals. As was done in the field of proton NMR studies of proteins, one way to overcome this resolution problem is to increase the magnetic field strength. Thus, it is proposed to extend our studies of cancer cell metabolism to diffusion-weighted proton NMR studies at 600 MHz. This becomes feasible because of the presence of a new 600 MHz instrument at Bar-Ilan University that is available for these studies (see attached letter). To our knowledge no proton NMR studies of cellular metabolism have been previously been reported at this high field strength. The signals one can expect to obtain information from are lactate and phospholipid precursors, such as are observed in brain spectra. However, so little work has been done in this area that the likelihood of seeing new and interesting phenomena is high.

Another advantage of this approach is that because of the higher sensitivity of proton NMR fewer cells will be needed to obtain spectra. The intrinsic sensitivity should give rise to approximately an order of magnitude improvement in the speed to obtain spectra (this is without considering the longer relaxation times for ^{31}P nor the extra times required for the diffusion weighting pulses in proton NMR). Since fewer cells are needed, the cost of the experiments will go down and many more experiments should be possible with fewer cells in less time. Another difference related to the difference in sensitivity is that ^{31}P NMR probes use 10 mm tubes and proton NMR probes generally use 5 mm tubes. But, previous attempts (unpublished) to build a 5 mm perfusion system, have encountered difficulties, largely due to the problem of obtaining water resistant seals in such small tubes. Also, the capillary tubing used to deliver and remove the perfusate takes up a considerable portion of the internal volume of the 5 mm tube, thus displacing many cells. Fortunately for us, the new 600 MHz instrument at Bar-Ilan University has an 8 mm proton probe that is equipped with a gradient system. This is a good compromise between the unnecessarily large size of 10 mm tubes for proton NMR studies and the too small size of 5 mm for construction of a workable perfusion system. Since all our previous work on cells using ^{31}P NMR has been done with 10 mm tubes, it will be necessary to develop an 8 mm perfusion system for cells. We will need to adapt our perfusion system to work at with 8 mm tubes, which should not be too difficult to accomplish.

C. Results

(i) Growth Curves in Matrigel with Hormones: The cell lines to be studied in this work are listed in Table 1. In order to determine the nature of the growth of these cell lines in matrigel and the effects of hormones and tamoxifen, a series of experiments were carried out in which cells were counted. The use of matrigel caused problems with automatic counting due to clumping of the cells, and the lack of complete degradation of matrigel by dispase. Ultimately, a commercial mixture of dispase and collagenase (Matrisperse) was found to work satisfactorily. The cells were counted manually. Growth curves of all cells were determined; Fig. 1 presents the curves for MCF-7, MIII and LCC2. The MIII cell line was selected for its anticipated responsiveness to estrogen and tamoxifen, while the LCC2 cell line was selected for its responsiveness to estrogen only. Upon treatment of these cell lines with these hormonal effectors the growth curves were not found to be significantly different. As a consequence no further hormonal growth studies were carried out with these cell lines.

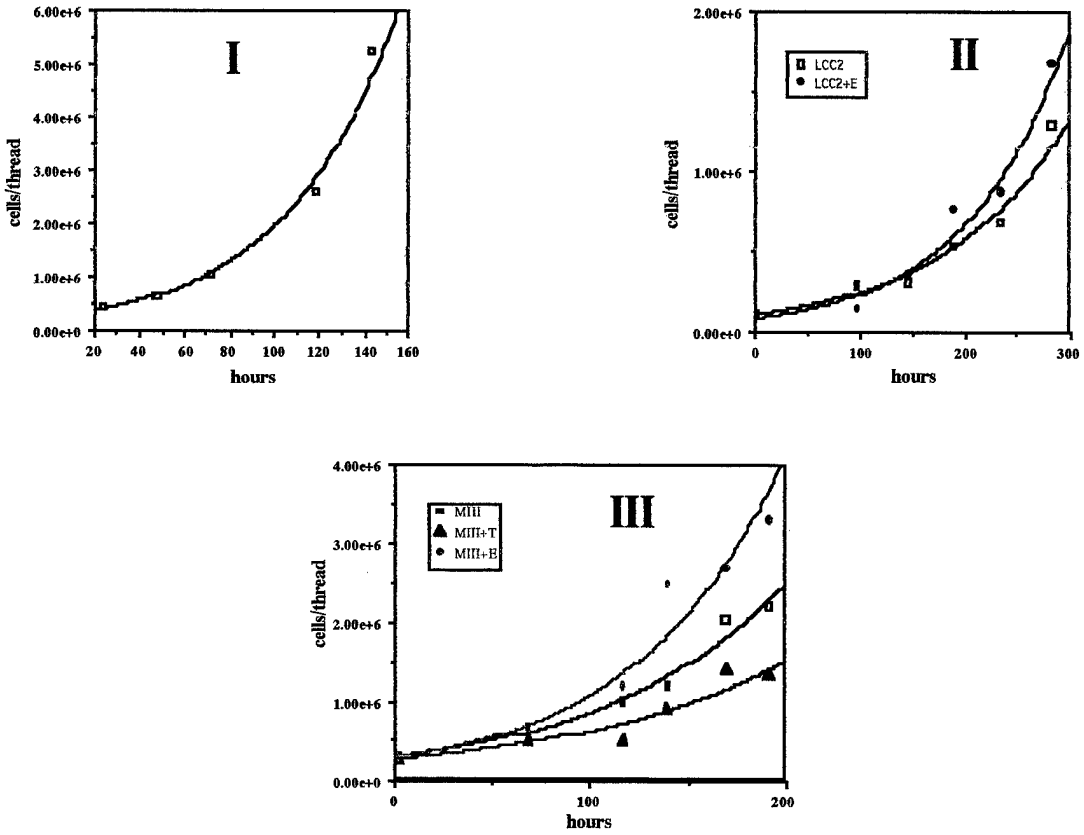


Fig. 1. Growth curves of cells growing in matrigel threads under standard culture conditions, including estradiol (E, 1 nM) and Tamoxifen (X, 0.5 μM) I - MCF-7, II - MCF-7/LCC2, III - MCF-7/MIII. All line fits have greater than 0.9 R confidence factor.

Table 1: Phenotypes of cells used.

Cell Line	ER	[1]Estrogen Dependence	[2]Estrogen Responsive	TAM Responsive	ICI 182,780 Responsive	Metastases
MCF-7	+ ve	dependent	responsive	sensitive	sensitive	no
MCF-7/MIII	+ ve	independent	responsive	sensitive	sensitive	yes
MCF-7/LCC2	+ ve	independent	responsive	resistant	sensitive	ND[3]
MCF-7/LY2	+ ve	responsive	resistant	resistant	resistant	NT[4]
MDA-MB231	- ve	independent	unrespons	resistant	resistant	yes
MDA-MB435	- ve	independent	unrespons	resistant	resistant	yes

1 = requirement for E2 to form tumors in nude mice; 2 = respond to E2 by inducing specific genes/mitogenesis; 3 = no data; 4 = non-tumorigenic.

(ii) Baseline Spectra of Cells: In previously studies baseline ^{31}P MR spectra of cell lines were obtained in perfused *agarose* gel threads (Ruiz-Cabello et al, 1994). Cells were routinely perfused for periods from 2 to 12 hours (Cohen et al, 1986; Ruiz-Cabello et al, 1994). The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that occurs after ca. 12-24 hours. These experiments were repeated multiple times (3 -5) with consistent results. Analysis of the results associated higher levels of PDE and UDPG and lower PC/GPC and PC/PE ratios with the acquisition of hormone independent status (Ruiz-Cabello et al, 1994).

These studies have now been performed for five of the six cell lines listed in Table 1 in *matrigel.* The cell line LY2 could not be grown satisfactorily during the period these experiments were underway. The ^{31}P spectra of each of these cell lines perfused in matrigel are shown in Fig. 2. The integrals of the resolved and assigned peaks normalized to the β-ATP peak are given in Fig 3.

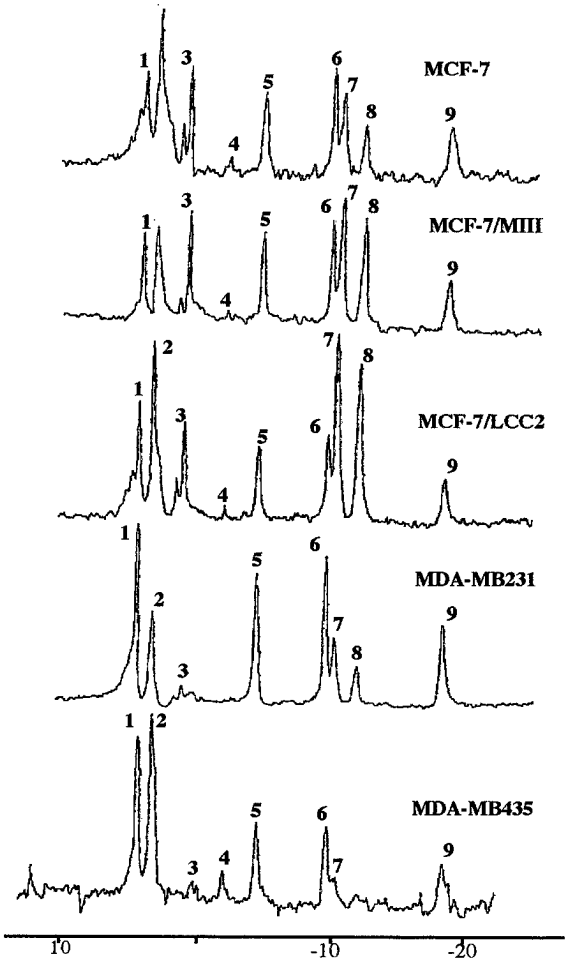


Fig. 2. 31P MR spectra of cell lines in matrigel threads perfused with the same medium. Assignments are: 1, PM; 2, Pi; 3, PD; 4, PCr; 5, g-ATP; 6, a-ATP; 7 & 8, UDPS & NADP; 9, b-ATP.

It should be noted that there are significant differences in these spectra, which constitute a "fingerprint" of the metabolic state of the given cell line. Specific large differences in metabolite levels might be considered related to the differences in their hormonal properties

(iii) Assignment of UDPS Compounds: The most significant difference observed in the baseline spectra is due to the diphosphodiester peak that varies widely from essentially unobservable in 435 cells to a high level in LCC2. This is listed as UDPS in Fig 3, since the nature of the sugar component is unknown. It is noted that the main difference in these cell lines is the presence or absence of ER and their consequent responsivity to estrogen. To assign the peaks responsible for the difference, extraction was performed as described previously. Spectra of extracts enable to resolve the different compounds in the UDPS area because the reduced line width. Peaks were assigned by spiking a spectrum with known compounds and were found to be UDP-N-acetylglucosamine (major component) and UDP-N-acetylgalactosamine (Fig. 4). Studies will be performed to elucidate the cause of this major metabolic difference between the cell lines.

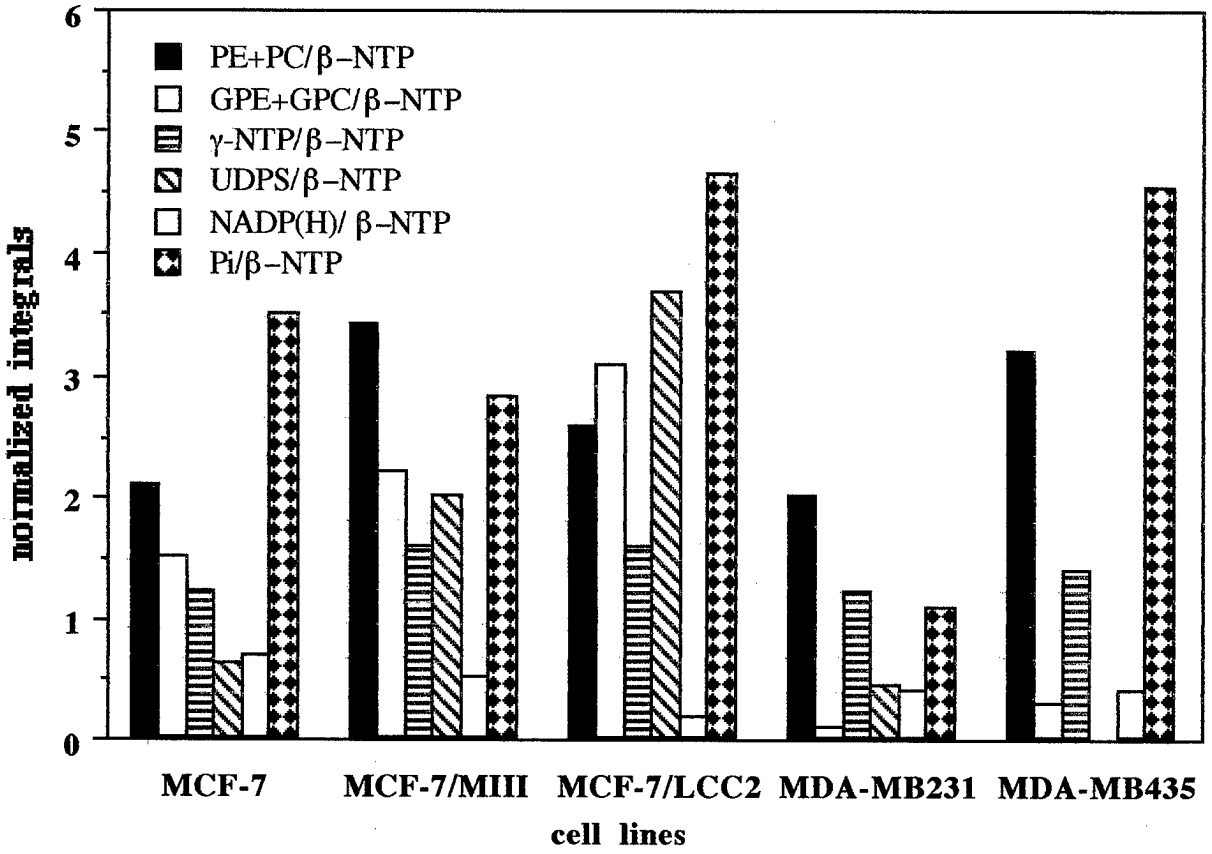


Fig. 3. Integration of 31P NMR signals from cell lines in Fig. 2, normalized to the β-ATP peak.

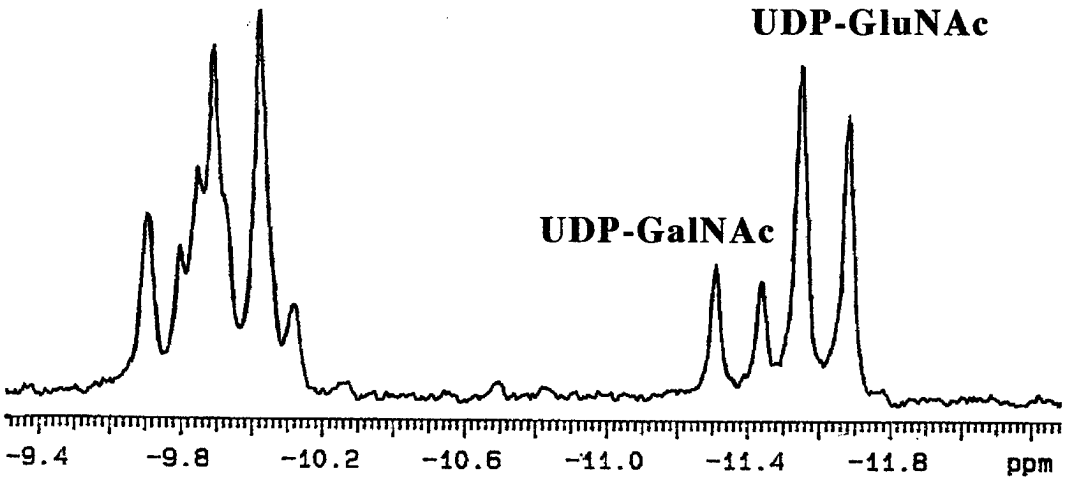


Fig. 4. Diphosphodiester region in the spectrum of MCF-7/LCC2 cells.

(iv) Characterization of Cell Cycle Distribution: The efficacy of hormone and drug action may depend on the phase of the cell cycle. Most of the effective anti-cancer drugs are cell cycle specific, that is to say their major effect is achieved at a specific stage in the cell cycle. We believe that studying the effects of hormones and drugs on these cell held at specific phase of the cell cycle may contribute to a better understanding of the correlation between drug action and a possible "metabolic fingerprint."

The cell cycle characteristics of each cell line were studied by Fluorescence Activated Cell Sorting (FACS). This flow cytometric method relies on a single time-measurement of both cell population and DNA content. Cells were fixed overnight with 80% ethanol, DNA was stained by incubating the cells with RNase A for 30 min at 37°C followed by incubation with Propidium Iodide and Triton for an additional 30 min at room temperature. MCF-7/LCC2 and MCF-7/MIII have reduced S phase levels, while MB-MDA-435 show much higher levels of cells in S phase (Fig. 5).

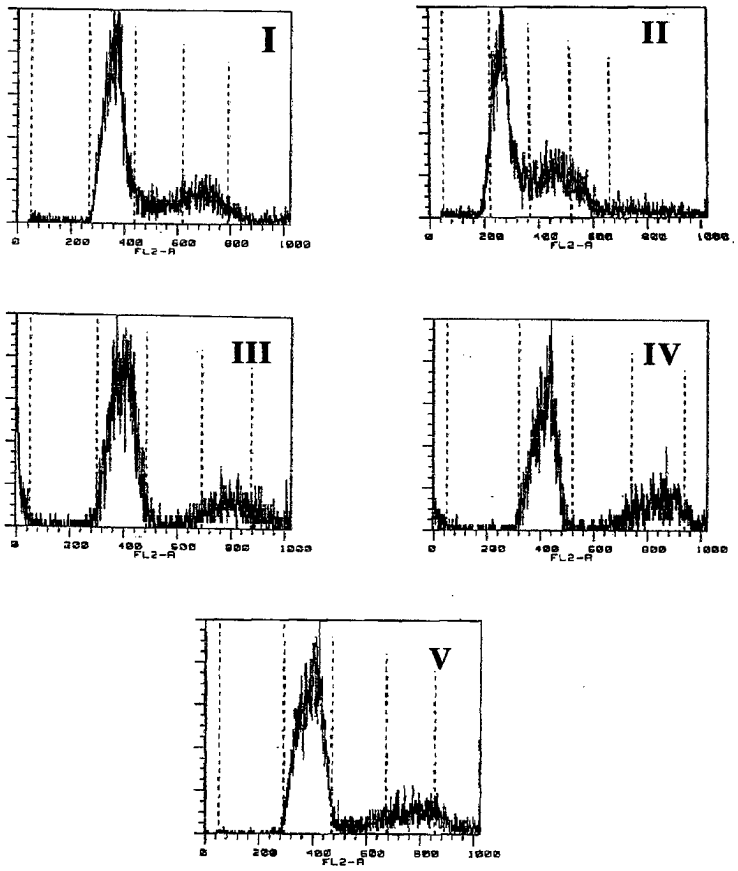


Fig. 5. Histograms showing cell cycle distributions of different cell lines (X-axis, DNA content; Y-axis, cell number).
I- MB-MDA-231 : G1 - 66.5%, S - 15.6%, G2/M 17.9%;
II- MB-MDA-435: G1 - 61%, S - 26.5%, G2/M - 12.5%;
III- MCF-7/LCC2: G1 - 76.5%, S - 6.1%, G2/M - 17.4%;
IV- MCF-7/MIII : G1 - 68.9%, S - 5.7%, G2/M - 25.3%;
V- MCF-7: G1 - 73.3%, S - 12.7 %, G2/M 14%.

To check whether growing in matrigel threads changes the cell cycle distribution, MB-MDA231 cells were grown in matrigel threads for three days. The cells were liberated from the matrigel by dissolving the threads with Matrisperse solution. The cell cycle distribution

was assayed by FACS, and was found to be comparable to the distributions found in previous experiments of cells grown in the culture dish. These differences in cell cycle phase distributions among the cell lines may be partly responsible for the difference in metabolite levels that were observed in the NMR spectra.

(v) Synchronization Experiments: Cells grown nearly to confluence, as those used for the NMR experiments, were shown to be 80-90% synchronized in G1. To check spectra of cells in other stages of the cell cycle, MDA-MB231 cells were synchronized in G2/M by incubating them for 12 h with 1mg/ml Nocodazole. This synchronization was reversible. Fig. 6 shows FACS analysis and integration results for control cells, cells synchronized by Nocodazole and cells recovering from Nocodazole treatment for 48h. Quantification was made by adding 1.5 M trimethyl phosphonoacetate to the extract. Significant decreases were seen in the ATP signal, the PC signal and other signals except for the PM region, which contains signals of PE and sugar phosphates. Recovery of PC was observed after 48 hours of incubation without Nocodazole.

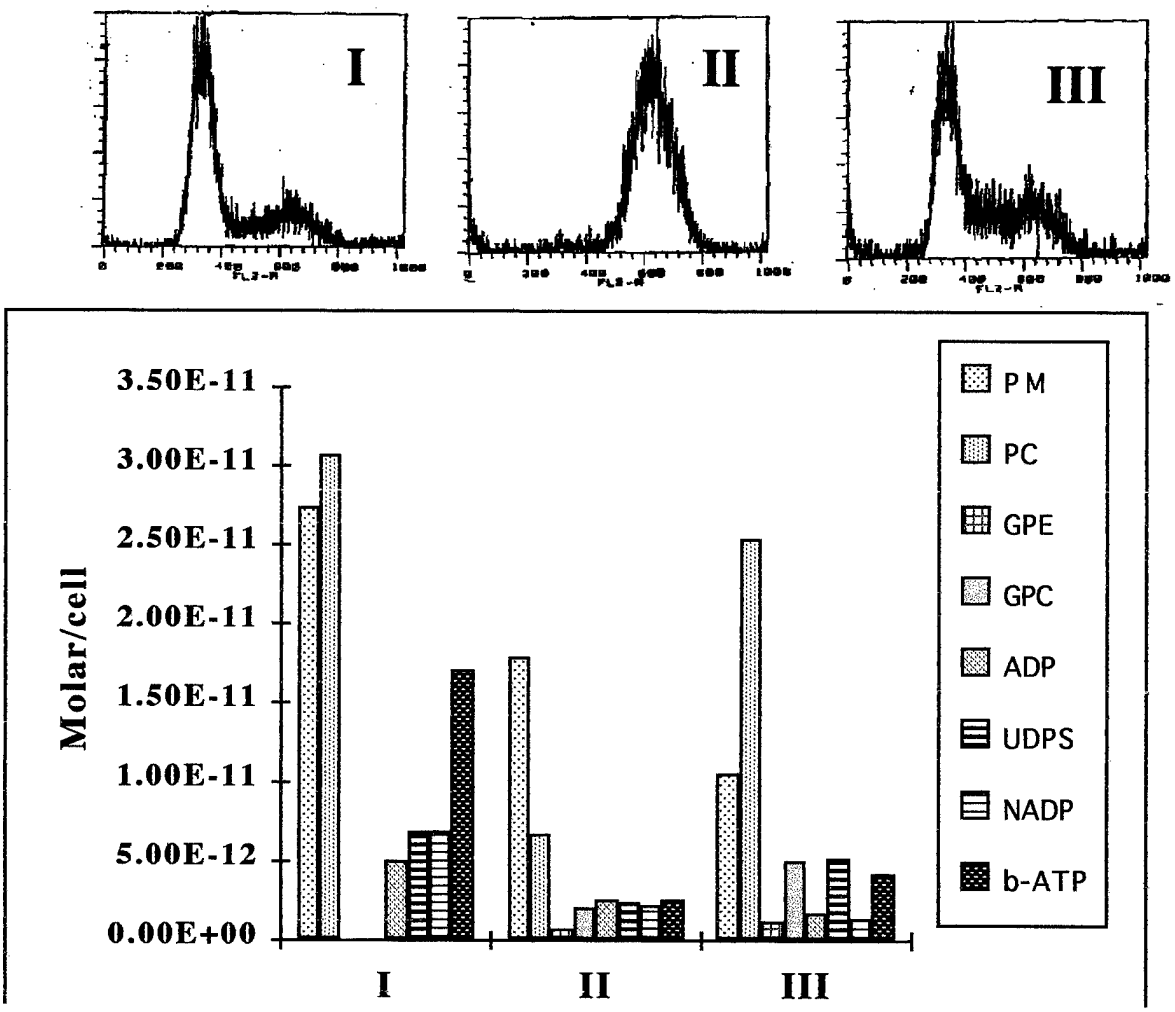


Fig. 6. Concentration of metabolites and cell cycle distribution for MB-MDA-231 line. I - control, II - treated for 12 h with 1mg/ml Nocodazole, III - after 48 hours of recovering from Nocodazole treatment (PM = phosphomonoester).

These preliminary results demonstrate that cells in different stages of cell cycle may have different levels of metabolites. (although long term effects of Nocodazole cannot be excluded). Several other techniques for cell synchronization which may exert minimal effect on cell characteristics are currently under study.

(vi) Effects of taxol: MB-MDA231 cells grown in culture were treated with $2.5x10^{-8}$M taxol For 48 hours, after which the cell content was extracted by perchloric acid treatment. ^{31}P NMR spectra from five experiments showed elevation of 60-100% in GPC signal and 19-60% in UDPS signal in cells treated with taxol, compared to untreated cells (the alteration in the phosphomonoester region was not very consistent in these experiments, although in most cases the level of PM increased upon taxol treatment). Fig. 7 presents the cellular intensities of metabolites in a representative experiment.

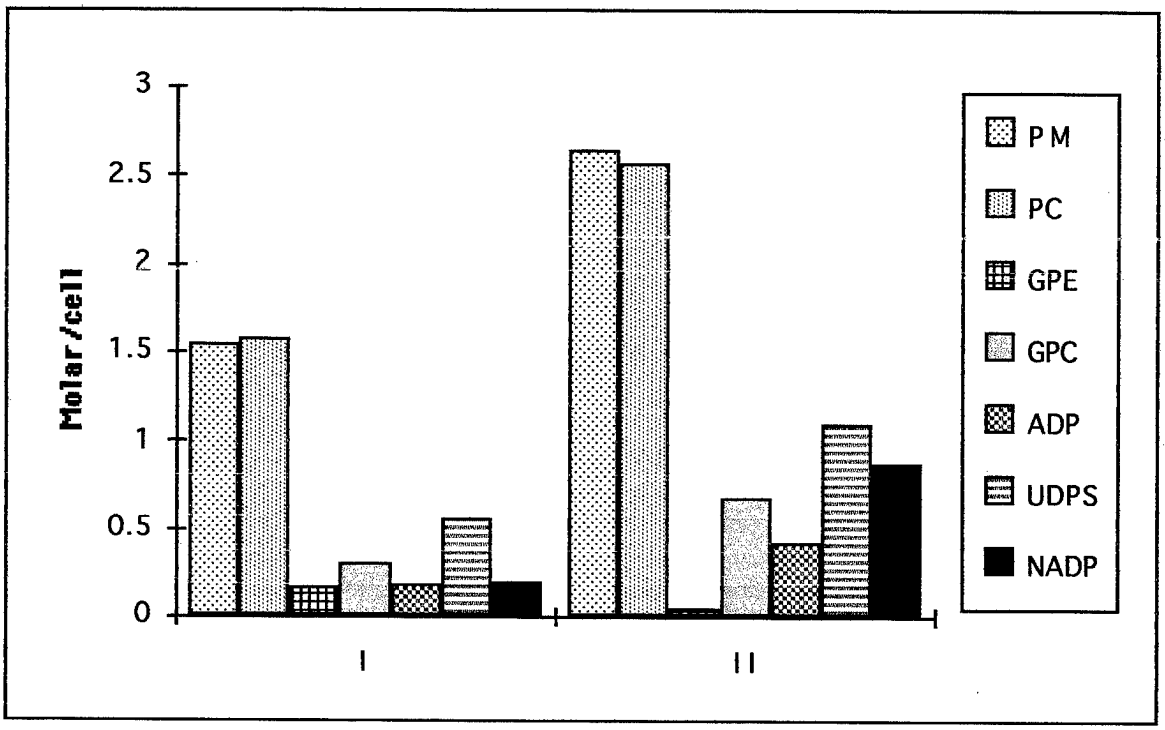


Fig. 7. Concentrations of metabolites of MB-MDA 231 cells. I - control, II- after 48 hours of treatment with 2.5e-8M taxol.

It can be seen that taxol has an effect on phosphate metabolites of cells, in particular it causes changes in the levels of GPC, which is a breakdown product of phospholipids and UDPS, which itself is an intermediate in the glycosylation pathway. The NMR study was accompanied by a study of the cell cycle components. FACS analysis revealed that taxol treatment induces a significant amount of apoptosis (as indicated by the sub-G1 levels of DNA) on the MB-MDA231 cells in a time-dependent manner (Fig. 8). This difference in cell phase content may correlates with the differences observed in the NMR studies. Cells checked by NMR and FACS were shown to be 97% viable by trypan blue exclusion.

The effects of taxol on cells grown in our *ex vivo* system (cells embedded in matrigel threads and perfused) are presented In Fig. 9. Slight inhibition of cell proliferation rate was observed for taxol treated cells (as indicated by the intensity of the β-ATP signal), and the level of PM was almost doubled. No significant increases in GPC or UDPS signals were observed. Followed the treatment with taxol, treads from the NMR tube were treated with matrisperse and the dispersed cells were analyzed by FACS. The amount of cells in apoptotic state was much less than that found for cells grown in culture dish. These results indicate

that different pattern of metabolites concentrations may correlate with different cell cycle distribution which results from altered experimental procedures.

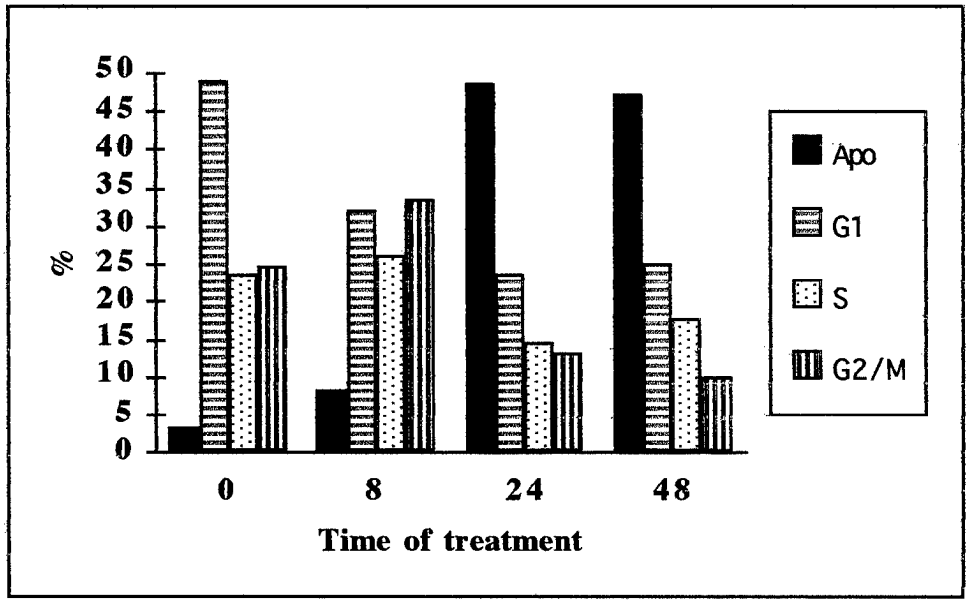
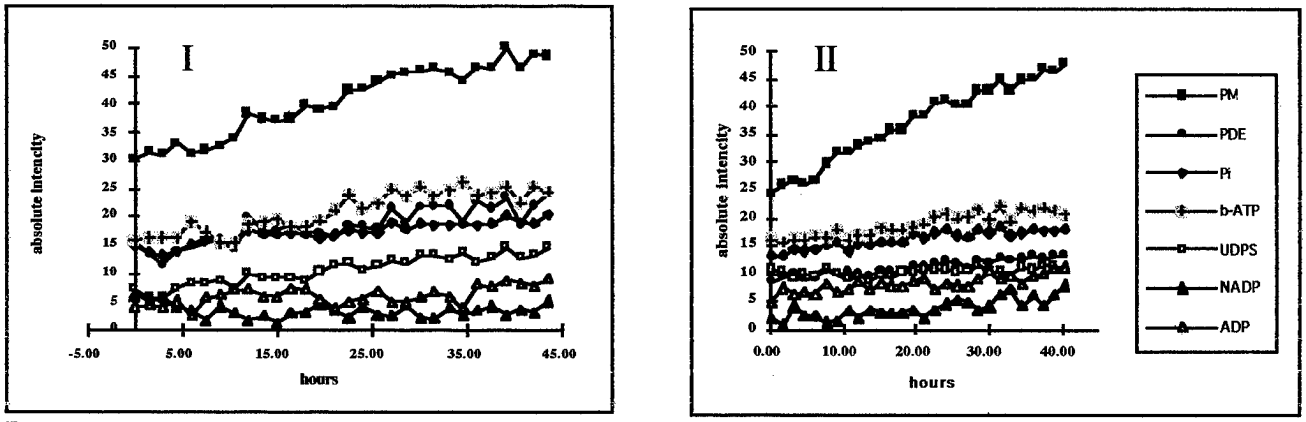


Fig. 8. Changes in cell cycle distribution and apoptosis during treatment with $2.5x10^{-8}$M taxol for 48 hours.

A.



B.

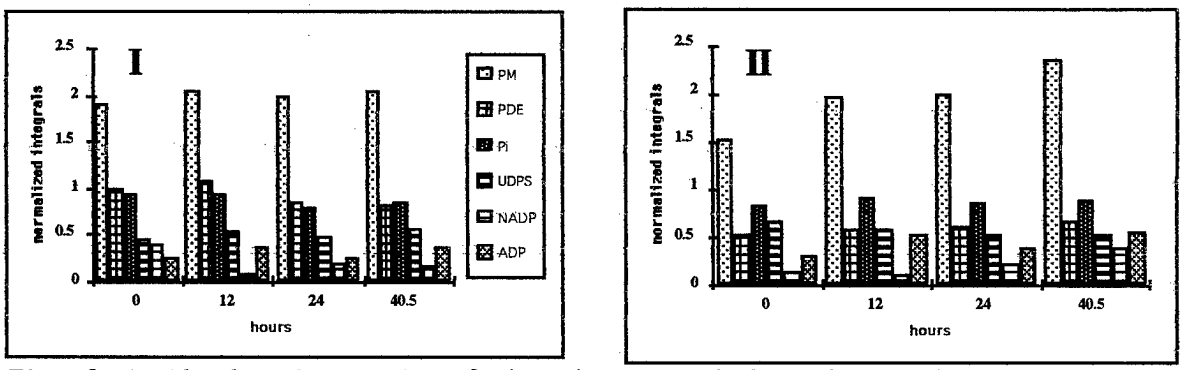


Fig. 9. A. Absolute intensities of phosphate metabolites during the *ex vivo* treatment with taxol. B. Integrals of metabolites normalized to β-ATP at different time points. I - control, II - perfused with $2.5x10^{-8}$M taxol.

Breast cancer cells have receptors for matrigel, and matrigel may affect several cellular processes. Nevertheless, growing cells in matrigel may be a better representation of the *in vivo* situation, and prospective *ex vivo* experiments will be performed on cells grown in matrigel at varied drug concentrations.

(vii) Effects of Methotrexate, Vinblastine, Vincristine and Adriamycin: Preliminary experiments were performed with these drugs (at concentrations in the 10^{-7} M range). MCF-7 and MBA-MB-231 cell lines were treated with methotrexate and several changes in metabolite intensities were recorded. The levels of GPC in MCF-7 and PM , DPDE and UDPS in MBA-MB-231 were elevated compared to the control. part of these changes may represent the effect of the drugs on phospholipid metabolism. UDPS levels were also found to be slightly higher in MBA-MB-231 cells treated with adriamycin or vincristine. No significant changes were observed for MBA-MB-231 cells treated with vinblastine. These studies are being expanded, and plans are to repeat the experiments in the presence of tamoxifen.

(viii) Proton NMR Studies of breast cancer cells: We intend to measure proton NMR spectra of perfused cells using the 600 MHz Bruker NMR spectrometer at Bar-Ilan University (10 mins drive from the Sheba campus; see attached letter). There are only two such high field machines in Israel, and this will give higher resolution than our previous instrument of 400 MHz. Higher resolution is desirable for proton NMR studies of cells, where there are many overlapping resonances of multiple metabolites. The cells will be cultured in our laboratory in Sheba Medical Center. In order to obtain spectra showing only intracellular metabolites, we will use a diffusion weighted NMR pulse sequence (van Zijl et al, 1991), that requires an applied magnetic field gradient in the order of 15-20 Gauss/cm. We are currently working with this instrument to implement this pulse sequence. The "txi8" 8mm probe at Bar-Ilan University is appropriate for these studies since it has a gradient that can reach values up to 50 Gauss/cm. However, the perfusion system we have used so far was built for a 10mm tube, therefore, we are now building a new insert for the 8mm tube to adapt the perfusion method for this probe and this instrument.

3. SUMMARY AND FUTURE PLANS

(a) We have found no significant effect of estrogen and tamoxifen on the growth curves of MIII cells and of estrogen on LCC2 cells grown in matrigel.

(b) We were able to carry out comparisons of baseline ^{31}P NMR spectra of 5 cell lines grown in matrigel.

(c) We have noted a significant difference in the UDPS concentrations in 435 and LCC2 cell lines. We will determine the relationship, if any, to the ER status of the particular cell lines.

(d) Differences were seen using FACS analysis between different cell lines in synchronization experiments initiated by Nocodazole. In future we will attempt to synchronize the cells by use of a centrifugal elutriator, a device for separating cells according to their diameter

(d) Differences were seen in spectra of extracts of MB-MDA-231 cells grown in plastic dishes, with and without taxol. However, studies carried out with this cell line grown embedded in matrigel threads and then extracted did not show the same differences. This indicates an important distinction between the two cellular environments, the nature of which will be pursued.

(e) No diffusion weighted proton NMR spectra have been obtained up to this point. However, these studies will be carried out in the final year of this project using the new 600 MHz NMR spectrometer at Bar-Ilan University (see attached letter). Progress is underway in implementing the diffusion weighted pulse sequence and adapting the perfusion system for the 8 mm probe on this instrument.

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5. Abbreviations Used:

Note that names of cells lines are not abbreviations.

^{31}P, phosphorus-31; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ER, estrogen receptor; GPC, glycerylphosphocholine; GPE, glycerylphosphoethanol-amine; MHz, megaHertz; MRI, magnetic resonance imaging; MRS, magnetic reson-ance spectroscopy; NADP, nicotine adenine diphosphate; NMR, nuclear magnetic resonance; PCr or PC, phosphocreatine; PD, phosphodiesters; Pi, inorganic phosphate; PM, phosphomonoesters; UDPG, uridine diphosphoglucose; UDPS, uridine diphosphosugar;